Cell
Leading Edge
Review

From Hype to Hope: The Gut Microbiota in Enteric Infectious Disease

Peter T. McKenney[1] and Eric G. Pamer[1,2,3,*]
[1]Immunology Program, Sloan Kettering Institute
[2]Lucille Castori Center for Microbes Inflammation and Cancer
[3]Infectious Diseases Service, Department of Medicine
Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
*Correspondence: pamere@mskcc.org
http://dx.doi.org/10.1016/j.cell.2015.11.032

One of the clearest functions of the gut microbiota in humans is resistance to colonization by enteric bacterial pathogens. Reconstitution of the microbiota offers an exciting therapeutic approach, but great challenges must be overcome.

We are all covered by and filled with bacteria. The first decade of sequence-based exploration of the human microbiome has established the importance of this dense and diverse ecosystem that we all carry. Entire fields of biology, such as physiology, immunology, and behavior, are in the process of realigning to account for the effects of the microbiota within their existing intellectual frameworks. In enteric infectious diseases, where the microbiota was generally believed to serve as a physical barrier to pathogens, our understanding of microbiota-mediated resistance has evolved considerably. It is now clear that the microbiota are active participants in preventing and sometimes in driving disease, depending on the state of the system. Work in the field has been driven by the idea that, if beneficial missing microbes are added back to the intestine, it may force out the offending microbes, rebalance the system, and prevent disease. Targeted approaches to rehabilitating the intestinal ecosystem, with fully defined indications, therapeutics, and diagnostics, may still be years away, but the remarkable success of early trials treating recurrent *Clostridium difficile* infection by reconstitution of the gut microbiota is cause for measured but realistic hope. Here, we will survey the known functions of the gut microbiota in defense against enteric infectious diseases with a focus on *C. difficile*, and we will address some of the major challenges facing those who hope to target the gut microbiota for therapy.

The Gut Microbiota

Germ-free laboratory animals can survive and reproduce in a sterile environment; however, the realities of life on a microbe-dominated planet led to the co-evolution of animals with bacteria (McFall-Ngai et al., 2013). Ever since the evolution of the coelomate digestive tract, microbial populations have co-evolved within the intestines of animal hosts to form a complex ecosystem: many bacterial taxa and their phages intermingle with a wide array of viruses, and a smaller number of fungi and archaea. The presence and abundance of protists and nematodes among the microbiota are likely to vary between populations, in part based on access to healthcare and local sanitation standards.

Although microbiologists have been isolating and studying gut bacteria since the beginnings of the discipline (Rajilić-Stojanović and de Vos, 2014), it is fair to say that pathogenic bacteria have received greater attention and are better understood than bacterial species that comprise the microbiota (Box 1). In the 1950s, antibiotics coupled with gnotobiotic mouse-rearing techniques led to the loss-of-function studies that established the functional importance of the microbiota in resistance to infections. During this period, it became clear that susceptibility to infection varied between hosts and that antibiotics can sensitize hosts to infection. In the 1970s, investigators determined the density of Enterococci and Enterobacteriaceae following various antibiotic treatments and demonstrated the importance of commensals in controlling human infections (Littman and Pamer, 2011; Smith et al., 2007).

Prior to the wide application of second-generation sequencing technology to analyze the microbiota, David Relman's group performed a seminal study characterizing the colonic microbiota of three healthy individuals by cloning and sequencing 13,355 bacterial 16S rRNA gene sequences. They found that many of the bacteria had never been cultivated, that most of the sequences derived from species had never been associated with human disease, and that each individual harbored a distinct colonic microbiota (Eckburg et al., 2005). In the last 10 years, high-throughput sequencing has prompted the adaptation of tools from microbial ecology to profile the entire gut microbiota population. This work has determined that the gut microbiota comprises hundreds of strains, the majority of which come from just two bacterial phyla: the Bacteroidetes and Firmicutes. Differences in microbiota composition have been noted between body sites and individuals and between cultures around the world. Knowledge of baseline compositions of the microbiota has linked changes in composition to phenotypes in laboratory models and pathology in humans. Infectious diseases, particularly hospital-acquired infections that follow antibiotic treatment, represent one of the clearest linkages between changes in the microbiota and health.

We will review four major functions of the microbiota that are relevant to bacterial infectious disease: direct inhibition, barrier maintenance, immune modulation, and metabolism (Figure 1). These functions all contribute to colonization resistance, defined

Box 1. Microbes, Hosts, and Damage

Terminology becomes complicated when incorporating the microbiota into microbial pathogenesis, which has focused narrowly on interactions between exogenous pathogens and hosts. The damage-response framework proposed by Pirofsky and Casadevall, which considers only microbes, hosts, and damage, is a method of fully integrating the microbiota into infectious disease (Casadevall and Pirofski, 2003, 2015). The source of damage can be microbes or host. Rather than defining individual microbes as pathogens, mutualists, or commensals, the damage-response framework focuses on the outcome of interactions between microbes and host. The state of the host becomes a function of all interactions with its microbes, resulting in net benefit, damage, or indifference. This allows the incorporation of host inflammatory feedforward loops that cause damage and exacerbate some acute infections (Stecher et al., 2007). The damage-response framework also pairs nicely with emerging concepts from immunology, such as disease tolerance, which is defined as host responses to infection that prevent damage while not directly lowering levels of the offending microbe (Medzhitov et al., 2012). Approaches that focus on outcomes rather than strict classification of microbes will be especially important in determining the roles of microbiota members in chronic diseases such as inflammatory bowel disease. The neutral term "microbiont," defined as any microbe living inside a host, has been proposed as an alternative to traditional host-centric classifications of microbes (Miles et al., 2015).

as the native ability of a host to suppress invasion by exogenous microorganisms (Stecher et al., 2013).

Direct Inhibition

Bacteria produce myriad bioactive small molecules and have long been the primary source of antibiotic candidates for the pharmaceutical industry (Milshteyn et al., 2014). One example is antimicrobial peptides, the bacteriocins, which selectively kill and inhibit the growth of competing bacteria (Kommineni et al., 2015). A computational prediction of biosynthetic gene clusters in the human microbiota identified >3,000 candidate clusters, including many newly annotated antimicrobial peptides, suggesting that these molecules may play a major role in shaping the population structure of the microbiota (Donia et al., 2014).

Bacteria also produce growth inhibitory molecules in secondary metabolism through modification of non-toxic host molecules, which has the potential for wide systemic effects (Box 2). Bile acids are one such example. They are produced in the liver and are secreted into the intestine in milligram quantities, where they are modified by the gut microbiota into dozens of different secondary bile acids, each with its own unique spectrum of chemical and biological activities (Devlin and Fischbach, 2015; Ridlon et al., 2006). Secondary bile acid production is drastically reduced by antibiotic treatment, after which mice and humans are susceptible to *C. difficile* infection (Buffie et al., 2015; Theriot et al., 2014; Weingarden et al., 2014). Reconstitution of antibiotic-treated mice with *C. scindens*, a bacterium capable of producing secondary bile acids, was sufficient to restore secondary bile acid levels and reduce *C. difficile* burden. Furthermore, levels of *C. scindens* were negatively correlated with *C. difficile* infection in antibiotic-treated hematopoietic stem cell transplant patients, suggesting that a similar mechanism may contribute to *C. difficile* resistance in humans (Buffie et al., 2015).

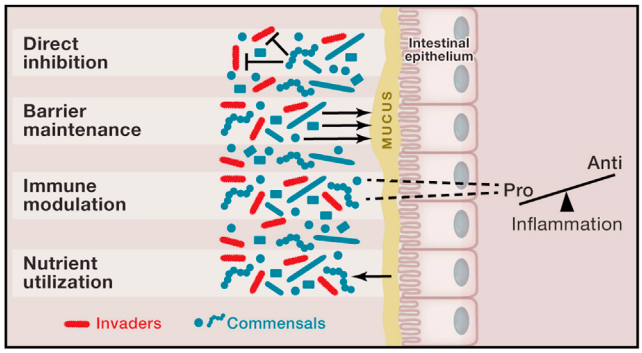

Figure 1. Colonization Resistance

The gut microbiome contributes to colonization resistance through at least four inter-related functions. (1) Direct inhibition of neighboring bacteria via the production of toxic compounds. (2) Maintenance of the mucus barrier and underlying intestinal epithelium. (3) Regulation of the immune response. (4) Efficient utilization of host nutrients, such as mucus polysaccharides, by commensals may serve to limit the expansion of less well-adapted invading microbes.

Barrier Maintenance

Gut bacteria facilitate their own sequestration within the intestine by regulating the strength of the intestinal barrier. The microbiota are restricted to the intestinal lumen by a mucus layer that overlays the intestinal epithelium and separates the microbiota from the patrolling immune cells of the intestinal lamina propria. Treatment of mice with antibiotics reduces the thickness of the mucus layer and results in increased contact between the underlying intestinal epithelium and gut bacteria (Wlodarska et al., 2011). Exposure to bacterial products is sufficient to stimulate mucus synthesis in germ-free mice (Johansson et al., 2015; Petersson et al., 2011), and alterations to host diet were sufficient to reduce the thickness of the mucus layer, further emphasizing the importance of microbial metabolism to barrier maintenance (Earle et al., 2015). Maintenance of the mucus barrier may be relevant clinically, as defects in mucus permeability have been observed in mouse mutants susceptible to colitis (Johansson et al., 2014). The microbiota directly affects the health of underlying intestinal epithelial cells by producing short-chain fatty acids (SCFAs), which are a primary nutrient for the colonic epithelium. Decreases in SCFAs may result in degradation of the epithelial barrier. In the absence of a strong epithelial barrier, *Fusobacterium necrophorum* and *Bacteroides fragilis*, common species among the microbiota, cause abscesses and tissue infections.

Immune Maturation and Inflammation

One of the first demonstrations that individual bacteria of the microbiota contribute to immune development came from studies with *Bacteroides fragilis*, which demonstrated that monocolonization of germ-free mice promoted development of CD4 T lymphocytes (Mazmanian et al., 2005). Subsequent studies have demonstrated that murine intestinal bacterial species promoted the differentiation of CD4 T cells into Th17 cells (Ivanov et al., 2009). Th17 cells can contribute to colonization resistance against pathogens but also contribute to the development of autoimmune pathology. Bacteria belonging to the cluster XIVa Clostridium group are associated with the development of anti-inflammatory T regulatory cells (Atarashi et al., 2013), as are bacterial consortia consisting of Bacteroides species (Faith et al.,

Box 2. The Microbiota as an Endocrine Organ

Bile acid metabolism by the microbiota has the potential to affect systemic physiology. Bile acids function as signaling molecules through multiple nuclear hormone receptors and G-protein-coupled receptors expressed in a variety of tissue types (Zhou and Hylemon, 2014) and cells of the immune system (Brestoff and Artis, 2013). Secondary bile acids tend to outcompete primary forms for binding to identified receptors. Therefore, the balance of primary to secondary bile acids has the potential to regulate signaling through these pathways (Ridlon and Bajaj, 2015). In addition, *C. scindens* may convert glucocorticoids (corticosteroids) to androgens; thus, this one bacterium may affect the balance of multiple classes of exogenous and endogenous steroid hormones (Ridlon et al., 2013). Microbial metabolites produced by spore-forming *Clostridia*, including bile acids, influence the levels of serotonin produced by enterochromaffin cells, suggesting that system-wide modulation of physiology and behavior may be mediated by the gut microbiota (Hsiao et al., 2013; Yano et al., 2015). Going forward, it will be important for the developers of orally dosed drug treatments to consider the effects of the microbiota on the bioavailability of drugs. The variability of the microbiota in the population may underlie some of the heterogeneity of treatment outcomes observed in clinical trials (Mani et al., 2014).

Box 3. Infectious Disease Ecology

Our deeper understanding of the microbiome's impact on the host forces us to reconsider infectious diseases from the perspective of interactions between species within a complex ecosystem. Infectious diseases are likely to occur by two major mechanisms: acquisition of an exogenous bacterium or expansion of an endogenous strain that causes harm at high levels (so-called pathobionts). *C. difficile* and other pathogens are carried asymptomatically in the population, and the question of how often cases are caused by de novo infection versus expansion remains a challenging exercise in epidemiology. Acquisition of a pathogen endemic to a hospital is conceptually similar to an invasive species. A foreign but well-adapted bacterium encounters a favorable environment (in this case, the GI tract of a patient made sensitive through antibiotics) and expands to fill the niche. The process of pathobiont expansion is similar to the loss of a predator, which allows expansion of former prey species. Mathematical models inspired by ecology and environmental engineering are being developed with increasing sophistication (Bucci and Xavier, 2014; Manor et al., 2014), and such models will be critical in guiding investigators in designing experiments in the laboratory and in testing hypotheses generated from model organisms on human sample sets (Buffie et al., 2015; Stein et al., 2013).

2014). Short-chain fatty acids derived from gut microbiota metabolism maintain the balance of inflammatory and anti-inflammatory T cell subsets (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). Thus, the balance of the bacterial population structure in the gut and the resulting metabolome have the potential to drive pro-inflammatory or anti-inflammatory responses to immune stimuli.

The microbiota is also a source of ligands for innate immune signaling. Treatment with antibiotics increases susceptibility of mice to dextran sodium sulfate (DSS)-mediated colitis, a phenotype that can be rescued by the administration of Toll-like receptor ligands. This suggests that pattern recognition receptor signaling originating from the microbiota is necessary for protection against epithelial damage (Rakoff-Nahoum et al., 2004). Intestinal microbes stimulate innate immune receptors to promote expression of bactericidal C-type lectins by intestinal epithelial cells that act directly to suppress bacterial growth (Vaishnava et al., 2011). Stimulation by intestinal bacteria can also enhance systemic antiviral responses (Abt et al., 2012; Ganal et al., 2012), while intestinal viruses can enhance antibacterial immunity (Kernbauer et al., 2014).

Utilization of Nutrients

Host nutrition is the primary source of energy for the gut microbiota, and changes in host dietary patterns can result in rapid changes in the population structure of the microbiota (Carmody et al., 2015; David et al., 2014). As nutrition shapes population structure and alters the gut metabolome, it may shift the microbiota to a state that is sensitive to infection (Ferreyra et al., 2014a). The ability to successfully colonize a mouse with a given bacterial strain has been correlated with abundance of close relatives in the target host (Stecher et al., 2010). These data suggest that high levels of a particular taxon may shift the gut metabolome to a state that is permissive to growth of closely related strains due to similar metabolic requirements. The microbiota can also produce nutrients that directly feed invading microbes. *Bacteroides thetaiotaomicron* can cleave sialic acid moieties from mucin glycan strands and produce high levels of succinate in mono-colonized gnotobiotic mice. The production of both of these metabolites by *B. thetaiotaomicron* increased the burden of *Clostridium difficile* in co-colonized mice (Ferreyra et al., 2014b; Ng et al., 2013). Such syntrophic interactions may be widespread between pathogens and the microbiota, and they represent potential targets for treatment (Box 3).

Enteric Infectious Diseases in the Post-microbiome Era

The impact of the full range of medical treatments on the microbiota remains largely unexplored. Prophylactic antibiotic administration often begins at birth, and the average child has many courses of antibiotics during development. Profiling of the microbiota in response to a single course of the commonly used antibiotic ciprofloxacin revealed that some changes to the microbiota were reversible within weeks, while others were permanent (Dethlefsen and Relman, 2011). Memory effects based on previous history have also been observed due to diet (Carmody et al., 2015), shifting of feeding time, (Zarrinpar et al., 2014), and sleep-wake cycles (Thaiss et al., 2014). The effects of diurnal rhythms on microbiota composition and immunity are likely to be significant, especially in light of recent reports of diurnal fluctuations in immune cell subsets and expression of immune signaling receptors in intestinal epithelial cells (Mukherji et al., 2013; Nguyen et al., 2013; Yu et al., 2013). Models derived from data at single time points may be missing key biology. All of these variables may contribute to the development of infectious disease through their effects on the microbiota and colonization resistance.

Treating Acute Infections

C. difficile is the most prominent microbiont exploiting antibiotic-mediated injury to the microbiota, with >250,000 cases annually. Cases of *C. difficile* are almost always associated with prior antibiotic treatment; thus, it seemed clear from the beginning that

susceptibility to this infection resulted from the loss of protective microbiota (Britton and Young, 2014). The importance of the microbiota is reflected in the ability of 16S sequencing data alone to distinguish between *C. difficile*-positive diarrhea, *C. difficile*-negative diarrhea, and controls (Schubert et al., 2014). If there were any doubts about the ability of microbiota reconstitution to cure recurrent *C. difficile* infection, they were put to rest with a randomized, controlled clinical trial of fecal microbiota transplantation (FMT) demonstrating >90% effectiveness, compared to 30% for conventional antibiotic treatment (van Nood et al., 2013). A recent meta-analysis suggests a cumulative cure rate of 89% in immunocompromised patients (Kelly et al., 2014). Mechanistic correlation between mouse and man came from an FMT study in humans that examined levels of bile acids during treatment, which found that levels of growth-inhibitory and spore-germination-inhibitory secondary bile acids were inversely correlated with sensitivity to *C. difficile* (Weingarden et al., 2014).

These data suggest that FMT represents a viable treatment option for patients with recurrent *C. difficile*. A fecal sample, banked before hospitalization for surgery or other planned interventions that involve antibiotics, may provide a method of preventing hospital-acquired infections while ensuring replenishment of the patient's own personalized microbiota. Such an approach would require an infrastructure of service labs to become economically viable. The relative effectiveness of donor stool transplants (heterologous) versus self-stool transplants (autologous) has not been tested, and it is possible that undesirable phenotypes may be transferred from donors to recipients (Alang and Kelly, 2015). The stability of the transferred microbiota over time is also unclear. Follow-up studies are beginning to appear that suggest that post-FMT variance in population structure of recipients is not significantly elevated over normal baseline variance in the donor (Fuentes et al., 2014; Weingarden et al., 2015).

Given the heterogeneous nature of fecal samples, resistance from clinicians treating chronically ill patients, and regulatory uncertainty, work has focused on defining the microbes responsible for colonization resistance versus *C. difficile*. Multiple labs have identified commensal microbes that confer resistance to *C. difficile* in germ-free (Reeves et al., 2012) and antibiotic-treated mice (Buffie et al., 2015; Lawley et al., 2012). Two of these studies identified strains from clostridial cluster XIVa as sufficient to reduce *C. difficile* burden. *C. scindens*, assigned to cluster XIVa, has the potential to directly inhibit *C. difficile* growth through production of growth-inhibitory and spore-germination-inhibitory secondary bile acids. Thus, *C. scindens* could form the base of a consortium designed to inhibit *C. difficile*. A mix of 17 clostridial strains—12 from cluster XIVa, including an isolate of *C. scindens*—induced the development of anti-inflammatory T regulatory (Treg) cells in mice and reduced colitis (Atarashi et al., 2013), as have combinations of Bacteroides (Faith et al., 2014). Inclusion of such strains could potentially synergize with *C. scindens* to reduce morbidity by suppressing the damage-producing inflammation caused by *C. difficile*.

In order to return an acutely infected patient to a state of health, it will be necessary for tailored treatments to both drive out the offending microbe and fully restore colonization resistance to a broad spectrum of damage-causing microbionts. While it is likely that single strains will be identified that can eliminate microbes of interest, it is unlikely that single strains will be sufficient to fully restore baseline levels of colonization resistance to patients that have lost significant proportions of the diversity of their microbiota.

Treating Chronic Dysbiosis

Patients undergoing allogeneic hematopoietic stem cell transplantation, which includes a regimen of antibiotic prophylaxis, often lose microbiota diversity and become dominated by antibiotic-resistant organisms such as *Streptococcus viridans*, *Enterococcus faecium*, and bacteria belonging to the Enterobacteriaceae family. And while patients can appear healthy with a low-diversity microbiota, the loss of microbiota diversity at the time of stem cell engraftment is associated with markedly increased mortality over 2 years following transplantation (Taur et al., 2012, 2014; Ubeda et al., 2010). In these cases, eliminating the dominant strains and restoring microbiota diversity would be desirable. In mouse models of *E. faecium* domination, FMT is effective in restoring diversity and eliminating *E. faceium* (Ubeda et al., 2013).

In order to eliminate unwanted strains in an otherwise healthy patient, it will be necessary for the donor microbiota to overcome the colonization resistance of the host and to expel the offending strain. This may not be possible in the absence of a treatment to lower colonization resistance. Colonization of germ-free mice with *B. fragilis* renders mice highly resistant to subsequent challenge with the same *B. fragilis* strain; however, mice mono-colonized with *B. fragilis* were not resistant to colonization by other closely related *Bacteroides* species. Furthermore, in these experiments, *B. fragilis* was neither expelled nor even reduced in CFU burden (Lee et al., 2013). These data suggest the existence of multiple distinct niches for closely related bacteria within the intestine and illustrate the challenges faced by prospective microbiome engineers in even simple distillations of the problem. Further investigations into the basic mechanisms of colonization and colonization resistance will be crucial to translating these ideas to the clinic.

Prospects

A simple Internet search returns links to do-it-yourself FMT advocacy web sites, instructional videos, and unsettling images of home blenders filled with brown liquid. This may have contributed to a recent decision by the U.S. Food and Drug Administration to regulate feces as a drug and to require an Investigational New Drug application for all applications of FMT, except in the case of severe recurrent *C. difficile*. FMT outside of a healthcare setting is not safe and should not be attempted. This statement may seem at odds with the tremendous success of FMT trials in treating *C. difficile*; however, the donors in these trials were carefully selected and screened in a process that eliminated >90% of candidates, and samples were processed according to Current Good Manufacturing Practices to minimize the potential of contamination (Petrof and Khoruts, 2014). A significant risk exists for transfer of pathogenic microbes and viruses, particularly in immune-compromised patients.

Engineering the microbiome for health is clearly desirable. Association studies have identified microbial candidates underlying

a diverse array of acute and chronic diseases. A major challenge going forward will be to determine efficient, minimally invasive methods of colonizing patients, coupled with the elimination of unwanted resident microbes. Methods of colonization may arise from studies aimed at understanding the phenomena that contribute to the stability of the microbiota (Lahti et al., 2014), and synthetic biology may uncover genetic modules that increase the efficiency of colonization (Yaung et al., 2015). Future laboratory investigations should include studies not only of colonization dynamics in germ-free and antibiotic-treated mice, but also of naive specific pathogen-free mice as well (Kommineni et al., 2015). Most useful would be the discovery of ''gain-of-function'' variants of intestinal bacteria that have the ability to outcompete and displace closely related strains in antibiotic naive humans. This will be a great challenge. More than 70 years of antibiotic chemistry have not produced a small molecule capable of selectively eliminating bacteria at the strain level; however, one of the world's densest microbial ecosystems is within us and may contain fantastic new molecules and genetic modules that have evolved for just this purpose. Designer microbial consortia have the potential to reduce the risks involved in FMT, but the challenges involved in their development are as great as with any other drug.

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